AMEA-nın Xəbərləri (biologiya və tibb elmləri), cild 69, №1, səh. 110-114 (2014)

Frequency of Occurrence Met235thr Human Angiotensinogene Mutant Variant in Patients with Cardiovascular Diseases

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Polymorphism of mutant variant of 235 (C-T) AGT gene was studied by means of complex of modern molecular-genetic methods of diagnosis for the first time in patients with cardio-vascular diseases. The mutation of 235 (C-T) in AGT gene was identified as 37.5% frequency of occurrence. Homozygous mutation type (T/T) was found in patients with severe arterial hypertension, while heterozygous mutation type (T/C) – in patients with severe as well as moderate arterial hypertension. Results of molecular- genetic investigations are of great practical importance. Timely revealing AGT mutation in gene 235 (C-T) will allow doctors to carry out prophylaxis in population with following qualified treatment of arterial hypertension.

***Key words**: genetic polymorphism, angiotensinogen, PCR, ischemic heart disease*

INTRODUCTION

Gene of angiotensin (AGT) encodes angiotensinogen protein which takes part in blood pressure and electrolytes balance control. When blood pressure lowers, angiotensinogen turns into angiotensin 1 under rennin effect, which in its turn becomes angiotensin 2 and raises the blood pressure, lessening the vessels diameter and increasing water and salts resorption (Kim et al., 2010). 15 point mutations, most of which lead to amino acids substitutions, were identified in the course of angiotensinogen gene study (Kurland et al., 2004). Much work was devoted to analysis of relations between these mutations and cardio-vascular diseases. Most attentively there were researched variants related to amino acidic substitutions as 235 (C-T) (Мет 235RThr) and 174 (C-T) (Thr174RMet). Substitution of thymine to cytosine in the position 235 of angiotensin gene (235 (C-T)) leads to methionine to threonine amino acid in-protein substitution. Analysis of 235 (C-T) angiotensinogen gene polymorphism sowed distinct correlation between 235 (C-T) polymorphism and various forms of hypertension, mainly, in European populations and in Japans. At the same time there observed the absence of the said association in Afro-Americans. It is also shown, that 235 (C-T) variant is an independent risk factor for myocardial infarct and ischemic heart disease development in Europeans, while in Japans there was identified no association of the given polymorphism with ischemic heart disease. Hence, the 235 (C-T) variant brings the certain pathogenic effect, but it couldn't be considered as a significant mutation, because its effect differs greatly in various ethnic groups' representatives (Gu et al., 2008). Effect of ethno-polymorphic variant can define non-equal link with some pathogenesis variants of angiotensinogen gene or, on the other hand, it can manifest itself on specific population genetic background only. Frequency of mutant gene variant is in range of 34-43%.Mutation inheritance type: autosome dominant (encounted in men and women equally, it is enough to inherit one mutant gene variant from any of parents, and at even chance children will get the disease). Indication to prescription to carry out the analysis is arterial hypertension and other cardio-vascular disease in the anamnesis of the patient or his close relatives, preparation to pregnancy, and to anti-hypertensive medications prescription (Пальцев, 2011; Нгуен, Шкурат, 2010; Liljedahi et al., 2003; Ash et al., 2010).

The goal of our researches is the study of 235 (C-T) gene of AGT and identification of frequency of the given polymorphism in patients with cardio-vascular diseases by means of molecular-genetic diagnosis methods.

The goal of our studies is the identification of angiotensinogen gene polymorphism 235 (C-T) presence with assessment of mutation phenotypic and gene frequencies in patients with cardiovascular diseases, taking into consideration blind spot in the studies related to 235 (C-T) polymorphism of angiotensinogen presence in population of the Republic.

MATERIALS AND METHODS

Material for the researches was 2ml of venous blood on EDTA (or heparin) anticoagulant from 24 people (13 men and 11 women) of 18 to 67 years of age with cardiovascular system disease.

Genomic DNA was isolated from venous blood using ready QIA ampgenomic DNA and RNA kits, manufactured by QIAGEN, Germany (Applicationinformation, 2000).

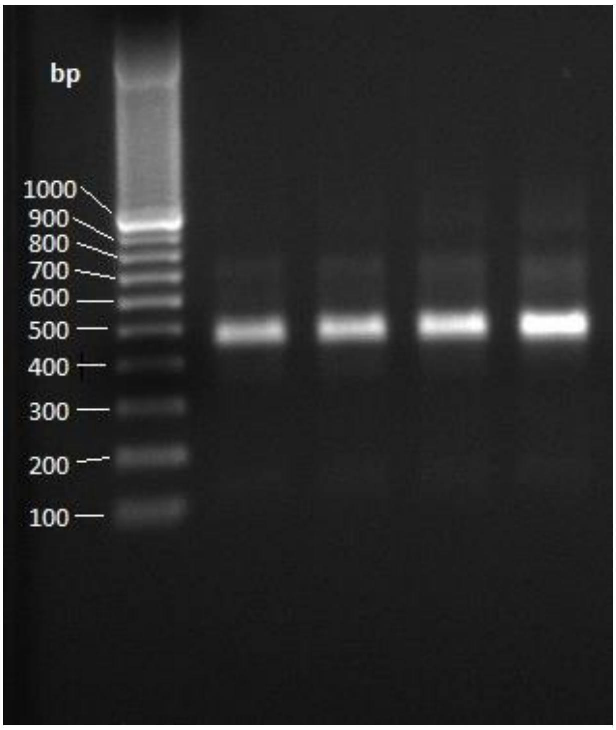
Isolation process was as follows: By means of automatic pipette volume 20µl we add an enzyme of QIAGEN Protease (or proteinase K) to Eppendorf tube of 1.5 ml. On top of that we add 200 µl of whole venous blood and 200 µl of AL buffer form QIAampgenomicDNAandRNA kits of QIAGEN Company. Mixture was stirred in a special device as pulse-vortex during 15 seconds. Then the tube was placed into thermostat at 56^0C for 10 minutes. After incubation is completed, 200 µl of ethanol (96-100%) was added into the content of the tube and stirred one more time in the pulse-vortex device during 15 seconds. After stirring the content of the tube was replaced into special micro-columns (QIAmpMinispincolumn) with toss-away tubes. Then the content of the micro-columns were centrifuged at 6000xg (8000 x rpm) during 1 minute at room temperature. The content of the tube after centrifuging was discarded, and micro-column with fixed genomic DNA was replaced into another tube-storage of 2ml volume. 500µl of AW1 buffer were added to column and centrifuged again at 6000xg (8000 x rpm) during 1 minute at room temperature. The content of the tube was discarded after centrifuging, and micro-column with fixed genomic DNA in it was also replaced into another tube-storage of 2ml of volume. Hence, 500µl of AW2 buffer were added and anew centrifuged, but in another regime: 20.000xg (14.000 x rpm) during 3 minutes at the room temperature. After substitution of a tube-storage the column was centrifuged again in the same regime during 1 minute. After complete remove of buffer AW2 from the column, the same column was placed into a regular tube with a cap for centrifuging and added 200µl of AE buffer from the kit or distilled water, specially prepared for molecular investigations, wait for a minute at room temperature (15-20^0C) and centrifuged also for a minute at 6000xg (8000 x rpm). After centrifuging the column was discarded, and the content of Eppendorf tube was used for the further investigations. Genomic DNA was stored in the fridge at -20^0C (Application information, 2000).

Intactness and amount of isolated genomic DNA as well as of the amplificate (gene fragment) after PCR were identified by electrophoresis in 1.7% agarose gel by means of electrophoretic device and power source (PowerPacBasicGelDocIMEZ) Imager, BioRad, USA. DNALadder 100 bp (Applicationinformation, 2000) was used as a marker for DNA synthesized fragments during electrophoresis.

Polymerase-chain reaction (PCR) was carried out in the following regime: 95oC for 2 minutes, (95oC for 30sec, 58oC for 30sec, 78oC for 2minutes for 25-30 cycles), 72oC for 10 minutes and pause at 4oC for 7 minutes in amplificator ProfessionalThermocycler manufactures by Biometra, Germany (3).

The quality of isolated genomic DNA from venous blood was checked in 1.7% agarose. 0,5xTAE buffer was used for electrophoresis. DNALadder 100bp was as a marker. Duration of electrophoresis is 30-45 minutes. After electrophoresis the agarose gel was placed into water solution of ethidium bromide for 5-10 minutes (10µl of ethidium bromide in 1 liter of distilled water). Then gel for genomic DNA development and taking photo was put into GelDoc^{TM}EZSystemInstallationGuide.

AGT gene was amplified when intact genomic DNA was isolated from blood. For that purpose two primers (Forward and Reverse) were used for each of five gene fragments. Five exons of AGT gene served as fragments for researches.



1 2 3 4 5

Figure 1. Electrophoregram of DNA size marker (band 1) and fragments of AGT gene after PCR (bands 2-5).

The structure of synthetic nucleotide primers which we used for PCR of five exons of AGT gene is presented in Table 1. Reagents for reaction mixture for PCR were added into eppendorf micro-tube in the following sequence: distilled water (used for molecular investigations) -30 µl, Buffer+$MgCl_2$ -8.2 µl, 2.5 µl of each primer (Forward and Reverse), dNTP - 1.25 µl,

Taq polymerase enzyme – 0.63 μl and genomic DNA - 5μl. After PCR quality and quantity of the amplificate were appraised by electrophoresis in 1.7% agarose. 0,5xTAE buffer was also used for electrophoresis of PCR products. DNA Ladder 100 bp was used as a marker. Duration of electrophoresis was 45-60 minutes. PCR fragments were stained with water solution of ethidium bromide. Staining duration was 5 minutes. PCR results were developed and shot in the device Gel Doc™ EZ System Installation Guide. Figure 1 presents an electrophoregram of DNA size marker shown as band 1 and fragments of AGT gene after PCR, which were isolated from patient‘s blood sample.

DNA fragments were purified after two consequent PCR using the following reagents: Agencourt AMPure XP и SPRI CleanSEQ Magnetic BEARDS, respectively. Nucleotide sequence of each of five fragments of AGT gene were identified by sequencing in GenomeLab CEQ and GeXP (Genetic Analysis Systems) manufactured by Beckman Coulter, USA.

RESULTS AND DISCUSSION

24 patients: 13 men and 11 women with cardio-vascular diseases in the age of 18 to 67 were included into researches. Identified duration of arterial hypertension was from 2 to 26 years according to anamnesis. Inheritance by arterial hypertension was burdened in 18 out of 24 patients (75%).

When rating severity degree of arterial hypertension, mild form of arterial hypertension was found in 6 patients out of 24 (25%), 10 (41.67%) of them had moderate arterial hypertension, and 8 (33.33%) had severe form of hypertension. The majority had a stable course of arterial hypertension. In 3 (12.5%) patients the course of arterial hypertension was crisis-like, those crises occurred not sooner than 3-4 times a year.

As a result of sequencing of AGT gene fragments, the substitution of cytosine nucleotide for thymine nucleotide in position 235 of the second intron, that leads to the substitution of methionine amino acid to threonine. In three cases: in two men and one woman, the said mutation was in homozygous state (T/T), in the rest six cases mutation was in heterozygous state (C/T).

Researches results of genetic polymorphism of AGT gene 235 (C-T) with identified phenotypic, genotypic and gene T and T alleles are presented in the Table 2.

As you see in the table, phenotypes (T/T), (C/T) and (C/C) frequencies are 12.5%, 25.0% and 62.5%, respectively. Genotypes frequency was equal to T/T – 0.1250, C/T – 0.2500 and C/C – 0.6250.

Therefore, frequencies of T and C alleles are 0.2500 and 0.7500, respectively. Phenotype frequency of 235 (C-T) polymorphism of AGT gene in the whole (T/T and C/T) became to be 37.5% and was similar to results obtained when researching populations of different countries throughout the world (2, 7, 8).

When appraising results on 235 (C-T) mutation among patients with different severity degrees off arterial hypertension, we’ve got the following picture: 6 of 8 (75%) patients with severe form of arterial hypertension have manifested the given mutation. Three (50%) of six patients with severe arterial hypertension had homozygous mutation state (T/T), and the rest three (50%) had heterozygous mutation (C/T).

Table 1. Name and structure of synthetic primers for AGT gene

N	Primer name	Nucleotide sequence of a primer
1	AGT F1	5′-TGC TTC TGT GTT TTC CCC AGT-3′
2	AGT R1	5′-AGA GAC AAG ACC GAG AAG GAG C-3′
3	AGT F2	5′-GGG CTA AAT GGT GAC AGG GA-3
4	AGT R2	5′-CCA GAG CCA GCA GAG AGG TTT-3′
5	AGT F3	5′-CCT CAT TCC TGC CCC TGT CT-3′
6	AGT R3	5′-GCT CAG GTG TGT CTA CTC CCC A-3′
7	AGT F4	5′-AGC ACA GAG GTC CTG AGC C-3′
8	AGT R4	5′-CCA AAG TCC AGG AAA GCA C-3′
9	AGT F5	5′-AGA TCA TAA GTC TTG GGC C-3′
10	AGT R4	5′-GCA TAG GCC AGG TTT CCA C-3′

Table 2. Phenotypic, genotypic and gene frequencies of T and C alleles of AGT gene

Phenotype frequency (in %)		Genotype frequency (in unit fractions)		Alleles frequency	
T/T	12,5	T/T	0,1250	T	0,2500
C/T	25,0	C/T	0,2500	C	0,7500
C/C	62,5	C/C	0,6250		

In three cases (30%) 235 (C-T) mutation was identified in patients group with moderate arterial hypertension. It should be mentioned that this mutation was absent in the group of patients with mild arterial hypertension.

Thus, we have researched 235 (C-T) polymorphism of AGT gene by means of complex of modern molecular genetics diagnostics methods for patients with cardio-vascular diseases. It has been identified the presence of the 235 (C-T) mutation of AGT gene with frequency of 37.5% for patients with cardio-vascular diseases. Homozygous mutation (T/T) has been found in patients with severe arterial hypertension. Heterozygous mutation (C/T) has been identified in people with severe arterial hypertension as well as in patients with moderate arterial hypertension. Consequently, the obtained molecular genetics researches' results in patients with cardio-vascular diseases have great practical importance. On-time revealing of 235 (C-T) mutation of AGT gene in patients will enable doctors to carry out prophylaxis with following professional treatment of arterial hypertension.

CONCLUSIONS

1. 235 (C-T) mutation of AGT gene presence has been identified in patients with cardio-vascular diseases and it has frequency of 37.5%.
2. In patients with severe arterial hypertension homozygous form of 235 (C-T) mutation frequency of occurance is as high as 75%.
3. The presence of 235 (C-T) mutation of AGT gene in heterozygous state (C/T) has been found in patients with severe and moderate arterial hypertension, while the given mutation hasn't been revealed in group with mild form of disease.

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Ürək Qan-Damar Sistemi Xəstəliklərində İnsanın Angiotenzinogen Geninin 235 (C-T) Mutant Variantının Rast Gəlmə Tezliyi

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Müasir molekulyar-genetik metodlar kompleksindən istifadə edərək ürək qan-damar sisteminin xəstəliklərində insanın angiotenzin geninin 235 (C-T) polimorfizmi tədqiq edilmişdir. 235 (C-T) mutasiyası tezliyi tədqiq edilən qrupda 37,5% təşkil etmişdir. Mutasiyanın homoziqot forması (T/T) arterial hipertenziyanın ağır olan qrupunda aşkar edilmişdir. Beləki, mutasiyanın heteroziqot formasına (T/C) arterial hipertenziyanın ağır və orta ağırlıqda olan qruplarında təsadüf edilmişdir. Molekulyar genetik tədqiqatların nəticəsinə angiotenzinogen geninin aşkarlanmış mutasiyasına 235 (C-T) əsaslanaraq əhali arasında arterial hipertenziyanın profilaktikası və ixtisaslı müalicəsi aparılacaqdır.

***Açar sözlər**: genetik polimorfizm, angiotenzinogen, PZR, ürəyin işemik xəstəliyi*

Частота Встречаемости Мутантного Варианта 235 (С-Т) Ангиотензиногена Человека У Больных Сердечно-Сосудистыми Заболеваниями

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У больных сердечно-сосудистыми заболеваниями изучен полиморфизм 235 (С-Т) гена АГТ с использованием комплекса современных молекулярно-генетических методов диагностики. Установлено наличие мутации 235 (С-Т) гена АГТ с частотой 37,5%. Гомозиготное состояние мутации (Т/Т) обнаружено у лиц с тяжелой формой артериальной гипертензии, тогда как гетерозиготное состояние мутации (Т/С) обнаружено у лиц с тяжелой и умеренной формой артериальной гипертензии. Полученные результаты молекулярно-генетических исследований имеют большое практическое значение. Своевременное выявление мутации 235 (С-Т) гена АГТ у населения позволит врачам проводить профилактику, а затем и квалифицированное лечение артериальной гипертензии.

Ключевые слова: *генетический полиморфизм, ангиотензиноген, ПЦР, ишемическая болезнь сердца*